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European Atomic Energy Community - EURATOM
Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek - T.N.O.

BONE MARROW TRANSPLANTATION IN IRRADIATED ANIMALS

PRODUCTION AND APPLICATION OF PATHOGEN FREE ANIMALS IN RADIATION EXPERIMENT

Annual Report 1964

1965

Work performed at the T.N.O.
Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek,
Rijswijk (Netherlands)

Association No. 029-63-1 BIAN

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BONE MARROW TRANSPLANTATION IN IRRADIATED ANIMALS
PRODUCTION AND APPLICATION OF PATHOGEN FREE ANIMALS IN RADIATION EXPERIMENTS *)

1 — BONE MARROW TRANSPLANTATION IN IRRADIATED ANIMALS

Experiments with monkeys

1.1 — X-disease

In 1962 an outbreak of a chronic disease occurred in the monkey colony, which soon affected the majority of the animals. The disease was characterized by hyperkeratosis, alopecia and wasting and run a slow course with a mortality of nearly 100 per cent. In the course of 1963 the disease was *identified* with a disease termed X-disease which has been described in young cattle, where it is due to the toxic effects of halogenated naphtalenes. The source of the toxic substance in the monkey colony was found to be sawdust, which was employed as an absorbant in the pans underneath the cages and which was consumed in small quantities by most monkeys. This sawdust is routinely steam sterilized before use. This use of sawdust was therefore discontinued and since that time no new cases of the disease have occurred spontaneously.

Experiments aimed at the isolation and identification of the toxic substance in the sawdust were continued during 1964. It was found that the sawdust becomes toxic as a result of the steam sterilization. Although chemical analyses of the sawdust demonstrated the presence of a high content of pentachlorophenol, the feeding or application of this substance did not induce the disease. However long continued feeding of a concentrated extract of autoclaved sawdust did produce mild symptoms of X-disease. In this stage of the investigation it was concluded that a further research on this subject fell outside the terms of the contract.

The costs of additional experiments to identify the toxic substance will be carried completely by the Radiobiological Institute. Autoclaving of sawdust is being increasingly employed for its use in specific pathogen free rodent colonies, so that the resumption of toxic by-products seems to be of more general significance.

1.2 — Fetal hemopoietic grafts

A considerable number of fetuses became available from the breeding colony. Hemopoietic cells from the liver of fetuses at the age of around 100 days were used for the restoration of lethally irradiated monkeys. It was soon found that the yield of cells from a fetus at that age ($0.3 — 1 \times 10^9$) is insufficient to cause repopulation of the recipient's hemopoietic tissues, so that it became necessary to pool the cells of several fetuses of the same age. This in turn necessitated the synchronization of pregnancies. A total of 5 experiments was performed with pooled fetal cells administered in numbers varying between 7 and 11×10^8 per kg body weight of the recipient. Regeneration of the hemopoiesis was observed only in the animal which received the highest cell dose. This animal developed secondary disease from which it succumbed 34 days

*) Manuscript received on July 15, 1965.

after transplantation. The other animals showed some temporarily regeneration at best and all died with bone marrow aplasia. These experiments are being continued with fresh as well as with stored cells as fetal material becomes available.

1.3 — Storage of hemopoietic cells at low temperatures

The investigations on the freezing and storage of autologous monkey bone marrow have been completed. Using the polyvinylpyrollidone-glycerol mixture to protect the cells during freezing, a total number of 15 irradiated monkeys has been treated with various numbers of preserved autologous cells. The minimum number of cells required for recovery was found to be 8×10^7/kg as compared to 4×10^7/kg in the case of fresh autologous bone marrow cells. This yields a preservation factor of 50 % under optimal conditions.

Freezing and storage with 10 % polyvinylpyrollidone which proved to give excellent protection to mouse bone marrow, was found to give disappointing results with monkey bone marrow. These results have again underlined the large differences between rodent and primate bone marrow. It is concluded that clinical bone marrow preservation methods should preferably be tested in monkeys. The polyvinylpyrollidone-glycerol procedure is recommended for clinical application since it was found to be the most effective technique in experiments with monkeys.

The investigation of freezing methods for use with fetal monkey liver cell suspensions are being continued on a limited scale.

1.4 — Pretreatment of the bone marrow graft

In mice several procedures of pretreating the homologous bone marrow have been found to result in a selective elimination of immunologically active cells, thereby causing a decreased incidence of secondary mortality in the recipients. Two of these procedures — freezing with glycerol and incubation at 37 °C for several hours — were investigated in pilot experiments with monkeys. The results were not encouraging. The best survival time (obtained following treatment with the frozen marrow) was 42 days but this animal died with overt signs of secondary disease. The experiments with monkeys were discontinued, awaiting the discovery of more effective methods in the mouse system.

1.5 — Pooled bone marrow

The experiments with pooled homologous bone marrow which were initiated in 1963, were completed. Table I shows that the number of pooled cells required to produce complete bone marrow regeneration is far greater than in the case of a single donor (2×10^8/kg). Following administration of the lower cell numbers bone marrow regeneration is poor and as a consequence secondary disease is much less pronounced. At least 10^9 pooled cells/kg seem to be required for adequate restoration of hemopoiesis but in these cases the usual lethal secondary disease ensues.

These results do not support the impression obtained from very limited clinical experience that pooling of homologous bone marrow decreases the risk of the development of secondary disease to a considerable extent.

1.6 — Chemical treatment of secondary disease

A systematic study has been made of the effects of treatment with methotrexate after the transplantation of homologous bone marrow in irradiated monkeys. Treatment with this drug has been found to be effective in mice in preventing secondary mortality by other investi-

gators. In the monkeys doses of 1/4 - 4 mg/day have been employed starting on days 1 - 10 following the bone marrow transplantation. Supportive treatment is attempted with parenteral fluids, blood transfusions and diets. The dosage schedules investigated so far were not effective in significantly prolonging survival but the experiments are being continued.

TABLE I

Treatment of irradiated monkeys with pooled bone marrow suspensions

Radiation dose 750 - 800 R (700 - 750 rad)

No. of cells/kg	No. of donors	No. of recipients	Survival time days	Secondary disease	Bone marrow regeneration
3.4×10^8	6	4	8–24	absent or slight	some
6.1×10^8	6	2	18	slight	some
7.3×10^8	5	1	16	absent	none
1.3×10^9	4	1	15	pronounced	good
2.4×10^9	5	1	16	pronounced	complete

1.7 — Selection of compatible donors

In the fields of bone marrow and organ transplantation it has become increasingly important to find the least incompatible host-donor combination. Blood group compatibility has generally been found to be of little significance. The finding of common antigens in leukocytes and nucleated cells from the tissues has stimulated attempts to type or group leukocytes in humans. Attempts are being made in several centres to demonstrate a parallelism between patterns of leukocyte antigens and of transplantation antigens, so as to enable recognition of comparative histocompatibility in humans.

Experiments along those lines have been initiated in 1964 with monkeys because they seem to be a suitable "second best" in such research. Many striking similarities have been observed between the reactions of patients and monkeys to homologous bone marrow transplantation after irradiation. It is expected that a significant decrease of the severity of secondary disease will be obtained when the degree of histoincompatibility between donor and host is diminished by selection.

Iso-antisera were produced by immunization of monkeys with skin grafts or blood from other monkeys. These antisera were strong enough to be used for the identification by agglutination of certain antigens on the leukocytes. By thus recording the antigen-pattern of the donor cells the take of an homologous bone marrow can be demonstrated.

For the recognition of relative histocompatibility however monospecific antisera of sufficient strength are required. Attempts are being made to produce these sera by absorbtion procedures and modifications of the technique of immunization.

A method of skin grafting suitable for monkeys was developed to use as an additional criterium in the evaluation of the degree of histocompatibility between individuals.

1.8 — Preirradiation sanitation of monkeys

A small number of baboons was studied with regard to suitability for radiation and bone marrow transplantation experiments. These animals were also heavily contaminated with strongyloides and did not seem to offer any advantages over the rhesus monkeys used thus far. At the time of writing a small group of Macacus speciosa monkeys is under investigation.

The treatment of oesophagostomum infections with Alcopar *) yielded satisfactory results. Trials are in progress with a new drug : Thiabendazole **), which seems to be superior.

Bone marrow transplantation in irradiated mice

1.9 — Specific immunological tolerance of the graft in radiation chimeras

In continuation of studies reported in the previous years investigations have been performed on the role of the thymus in the development of immunity and of specific tolerance in radiation chimeras. In the absence of the thymus the return of anti-third party activity is greatly prolonged but — depending on the host-donor combination used — some restoration of this function was eventually observed. In these cases the graft remained tolerant towards the host, from which it was concluded that the thymus is not wholly essential for the development of specific immunological tolerance.

Attempts have been initiated to characterize the factors in the thymus which are responsible for restoration of normal immunological reactivity in radiation chimeras by the use of diffusion chambers containing fragments of the thymus of newborn mice. So far implantation of such chambers remained without significant effects. The suitability of the diffusion chambers for this type of experiment is being investigated.

The distribution of labeled hemopoietic cells in normal and thymectomized animals has been studied with autoradiographic techniques. No evidence of a passage of these cells through the thymus following whole body irradiation and hemopoietic cell transplantation was obtained. The latter investigations have been concluded.

Studies on the persistence of immunological tolerance following transfer of tolerant cells into irradiated hosts have been continued. In addition to the role of the thymus in the maintainance of tolerance, the effects of variations on the numbers of passaged bone marrow cells have been investigated. It has been concluded that maintainance of tolerance within a lymphatic cell population is independent of an antigen excess and that loss of tolerance within an intact organism is due to replacement of the original tolerant lymphatic cell population by new cells derived from bone marrow precursor cells. The latter replacement requires the presence of the thymus. The capacity of the bone marrow in radiation chimeras to supply these precursor cells is dependent on the number of cells injected into the first host.

1.10 — Immunological techniques

A semi-quantitative test was developed to measure the antibody synthesis of individual cells of the lymphatic tissues *in vitro*. Lymphoid cell suspensions derived from animals immunized with sheep erythrocytes were employed. The possibility of using the test system for the detection of other antibodies by employing antigen-coated sheep erythrocytes is being investigated.

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*) (2-(4'-thiazolyl)-benzimidazole)

**) benzyldimethyl-(β-phenoxyethyl) ammonium embonate

1.11 — Factors influencing the proliferation of foreign bone marrow cells in irradiated hosts

The acute killing effect of homologous lymphoid cells in irradiated animals has been studied in relation to the number of bone marrow cells transplanted simultaneously.

The proliferative capacity of hemopoietic cells derived from radiation chimeras was found to be different from that of normal hemopoietic cells in that the capacity to repopulate the lymphatic tissues of irradiated (second) recipients was permanently impaired. This change was dependent on the number of bone marrow cells used to restore the first host.

A comparative study of the repopulation of the hemopoietic tissues in mice following partial body irradiation and whole body irradiation plus isologous bone marrow transplantation was made : three different techniques were employed. Survival of irradiated mice, rate of repopulation of the hemopoietic tissues and the colony formation in spleen. Injected hemopoietic cells were consistently found to be much more effective (by a factor of the order of 100) than cells shielded *in situ*. These experiments are being concluded.

1.12 — Secondary disease

The similarity between the post-thymectomy wasting syndrome and secondary disease following homologous or heterologous bone marrow transplantation in mice was confirmed by extensive histological studies of the two syndromes. In contrast to earlier observations by others it was found that the lymphatic atrophy which occurs in the thymectomized animals is of a secondary nature rather than primary. The thymectomized animals develop in addition a number of lesions which are considered as highly characteristic for certain auto-immune diseases in humans. On the basis of these findings it has been postulated that the post-thymectomy wasting syndrome is the result of an auto-immune reaction which develops in the absence of the thymus during the neonatal period. Confirmation of this hypothesis has been sought by using a variety of serological and transplantation techniques. A large number of positive Coombs reactions have been found among wasting thymectomized C57BL mice but not among mice of other strains. The nature of the antibodies involved is under investigation.

A comparison of the histopathological lesions of secondary disease, the post-thymectomy wasting syndrome and of NZB mice (which suffer from spontaneous auto-immune disease) is being made. In addition investigations are in progress concerning the presence of auto-immune factors in the secondary disease which develops under certain conditions following lethal irradiation and *isologous* bone marrow transplantation.

Experiments have been started with heterologous bone marrow transplantation into lethally irradiated germfree mice. The purpose of these investigations is to study the histopathological lesions accompanying secondary disease under germfree conditions to allow an evaluation of the contribution of infections to the syndrome.

1.13 — Prevention of secondary disease

A screening system was developed previously for the testing of methods aimed at the selective elimination of immunologically competent cells from suspensions *in vitro*. This system was used to investigate several new methods described by the group of Mathé. One consists of freezing and storage of the suspensions at low temperature. In our system there was some indication of selective elimination of immune cells but the effect was not impressive. The second procedure consists of incubation of the suspension for 1 or 2 hours at 37 °C and this was found to be highly effective in some experiments. Unfortunately this effect was not reproducible. The causes of this variation are being investigated.

1.14 — Treatment of secondary disease

Several degrees of secondary disease can be reproducibly induced in F_1 hybrid mice by whole body irradiation and the transplantation of mixtures of bone marrow and spleen from parent strain donors.

In these mice various dosage schedules of methotrexate have been studied. The results will be compared with the effects of methotrexate on secondary disease in monkeys. It is intended to employ such a system for the evaluation of other immunosuppressive drugs with respect to their capacity to modify the severity of secondary disease.

2 — PRODUCTION AND EXPERIMENTAL EVALUATION OF PATHOGEN FREE ANIMALS

2.1 — Specific pathogen free rats

A new SPF rat colony was started in the second half of 1964. This colony is being serviced by caretakers working in a plastic suit with air supply which provides a complete barrier between the personnel and the animals. The suit is desinfected on the outside before each entry into the colony. Rats from several inbred strains are derived by caesarian section, introduced aseptically into the colony building and fostered by gnotobiotic females that were kept previously in isolators. The microflora of the rats in this protected colony is being consistently analyzed to provide information on the efficiency of the barrier system. Work is continuing on modifications and improvements of the personnel suits.

2.2 — Gnotobionts

Satisfactory progress has been made with the germfree mouse colony. Breeding of the ND2 mice was excellent and many animals were available for experimental use during this year.

Small groups of germfree mice have been supplied to other laboratories in the Netherlands for the testing of their new germfree installations or for the establishment of an SPF mouse colony. Among these institutes were the Netherlands Cancer Institute, Amsterdam; the National Institute of Health, Utrecht, and the Laboratory Animal Farm TNO, Zeist. A group of gnotobiotic rats was supplied to the latter farm for the establishment of its SPF rat colony.

The first attempts to introduce mice of the CBA and C57BL strains into the germfree state failed but recent trials have been more promising.

Germfree rats obtained early in 1964 from elsewhere have failed to reproduce. Another nucleus of germfree rats will be acquired as soon as possible.

2.3 — Enterobacteriaceae free mice

A program has been started to produce and maintain mice which are completely free of Enterobacteria. These mice are required for the study of the effects of whole body neutron irradiation because such experiments are constantly disturbed by fatal septicemia caused by intestinal microorganisms e.g. proteus. Among the methods employed for the production of these mice are prolonged treatment with the antibiotic Kanamycin in a closed isolator system and contamination of germfree mice with a specified microflora in a less protected environment.

So far normal reproduction has occurred but several difficulties have been encountered in the raising of the young. Attempts are in progress to overcome these difficulties by modifications of the autoclaved diet.

2.4 — Irradiation experiments

Neutron and γ irradiations are being carried out with Enterobacteria free mice under continuous bacteriological supervision. The radiosensitivities of germfree and conventional mice have been compared. The results are in accordance with those obtained at Lobund Institute. The survival following doses of X irradiation above 1,200 rad is significantly prolonged in the germfree mice. The effect of bone marrow transplantation following these high doses of irradiation is under investigation. A detailed histological study is in progress of the tissues of normal and irradiated germfree mice with emphasis on the intestinal tract.

LIST OF PAPERS PUBLISHED IN 1964

H. BALNER, M.J. DE VRIES and D.W. VAN BEKKUM.
Secondary disease in rat radiation chimeras.
J. Nat. Cancer Inst. **32**, 419-459, 1964.

H. BALNER.
Persistence of tolerance towards donor-type antigens after temporary chimerism in rats.
Transplantation **2**, 464-474, 1964.

D.W. VAN BEKKUM.
The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures.
I. Effect of storage at 4°.

D.W. VAN BEKKUM, L.M. VAN PUTTEN and M.J. DE VRIES.
Identification of causative factors of X-disease in monkeys.
Laboratory Primate Newsletter **3**, 1-2, 1964.

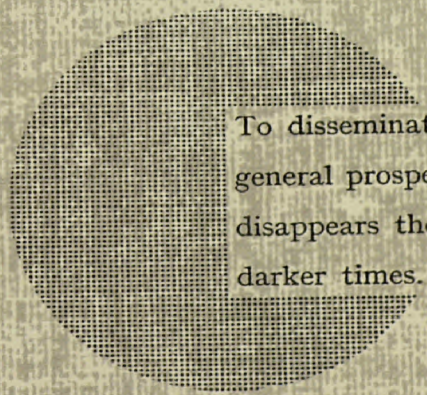
O. BROCADES ZAALBERG.
A simple method to detect single antibody forming cells.
Nature **202**, 1231, 1964.

L.M. VAN PUTTEN.
Thymectomy : Effect on secondary disease in radiation chimeras.
Science **145**, art. 1, 96-113, 1964.

L.M. VAN PUTTEN.
The effectiveness of different freeze storage techniques for mouse bone marrow cell suspensions.
Ann. N.Y. Acad. Sci. **114**, art. 1, 695-700, 1964.

O. VOS.
Transplantation of various numbers of foreign bone marrow cells in mice after lethal and supralethal X-irradiation.
Int. J. Rad. Biol. **8**, 21-33, 1964.

M.J. DE VRIES, L.M. VAN PUTTEN, H. BALNER and D.W. VAN BEKKUM.
Lésions suggérant une réactivité autoimmune chez des souris atteintes de la "Runt disease" après thymectomie néonatale.
Rev. Franc. Etudes Clin. Biol. **9**, 381-397, 1964.



To disseminate knowledge is to disseminate prosperity — I mean general prosperity and not individual riches — and with prosperity disappears the greater part of the evil which is our heritage from darker times.

Alfred Nobel

EURATOM — C.I.D.
51-53, rue Belliard
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